1 **Computational evidence of a new allosteric communication**

2 **pathway between active sites and putative regulatory sites in the**

3 **alanine racemase of *Mycobacterium tuberculosis***

4 Short title: Dynamics of alanine racemases reveal new allosteric sites in *M. tb*

5 Alr

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Abstract

23   Alanine racemase, a popular drug target from *Mycobacterium tuberculosis,*

24   catalyzes the biosynthesis of D-alanine, an essential component in bacterial cell walls. With

25   the help of elastic network models of alanine racemase from *Mycobacterium tuberculosis*, we

26   show that the mycobacterial enzyme fluctuates between two undiscovered states—a closed

27   and an open state. A previous experimental screen identified several drug-like lead

28   compounds against the mycobacterial alanine racemase, whose inhibitory mechanisms are

29   not known. Docking simulations of the inhibitor leads onto the mycobacterial enzyme

30   conformations obtained from the dynamics of the enzyme provide first clues to a putative

31   regulatory role for two new pockets targeted by the leads. Further, our results implicate the

32   movements of a short helix, behind the communication between the new pockets and the

33   active site, indicating allosteric mechanisms for the inhibition. Based on our findings, we

34   theorize that catalysis is feasible only in the open state. The putative regulatory pockets and

35   the enzyme fluctuations are conserved across several alanine racemase homologs from

36   diverse bacterial species, mostly pathogenic, pointing to a common regulatory mechanism

37   important in drug discovery.

Author summary

39   In spite of the discovery of many inhibitors against the TB-causing pathogen

40   *Mycobacterium tuberculosis*, only a very few have reached the market as effective TB drugs.

41   Most of the marketed TB drugs induce toxic side effects in patients, as they non-specifically

42   target human cells in addition to pathogens. One such TB drug, D-cycloserine, targets

43   pyridoxal phosphate moiety non-specifically regardless of whether it is present in the

44   pathogen or the human host enzymes. D-cycloserine was developed to inactivate alanine

45   racemase in TB causing pathogen. Alanine racemase is a bacterial enzyme essential in cell

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46   wall synthesis. Serious side effects caused by TB drugs like D-cycloserine, lead to patients'

47   non-compliance with treatment regimen, often causing fatal outcomes. Current drug

48   discovery efforts focus on finding specific, non-toxic TB drugs. Through computational

49   studies, we have identified new pockets on the mycobacterial alanine racemase and show that

50   they can bind drug-like compounds. The location of these pockets away from the pyridoxal

51   phosphate-containing active site, make them attractive target sites for novel, specific TB

52   drugs. We demonstrate the presence of these pockets in alanine racemases from several

53   pathogens and expect our findings to accelerate the discovery of non-toxic drugs against TB

54   and other bacterial infections.

Introduction

56   Tuberculosis is one of the top 10 causes of mortality globally and according

57   to latest available estimates, 10.4 million people developed this disease in 2016, of which 4.9

58   million people were infected with multidrug-resistant TB strains (MDR-TB) [1]. The

59   prevalence of multidrug-resistant TB (MDR-TB) and extensively drug-resistant tuberculosis

60   (XDR-TB) necessitates the inclusion of novel anti-tubercular therapies and strategies in the

61   treatment of TB. Treatment regimen comprising simultaneous use of multiple drugs is the

62   current strategy in practice [2]. Despite the implementation of this strategy, TB mortality

63   rates have not abated. Therefore, efforts to eradicate the TB pandemic have been stepped up

64   globally through research oriented towards finding new drugs against the tubercle bacilli [3].

65   Alanine racemase (EC 5.1.1.1; Alr), an essential bacterial enzyme [4] is a

66   popular drug target due to the absence of human homologs. The enzyme catalyzes the inter-

67   conversion of L- and D-alanine and requires pyridoxal 5'-phosphate (PLP) as a cofactor. PLP

68   is covalently attached to the enzyme through an internal Schiff's base linkage [5]. In the L to

69   D direction, the enzyme catalyzes the formation of D-alanine, an essential component of D-

70   alanyl-D-alanine found in the peptidoglycan layer in bacterial cell walls [5]. In some bacteria

71   including *Escherichia coli* [6], *Salmonella typhimurium* [7] and *Pseudomonas aeruginosa*

72   [8], there are two Alr isozymes (Alr1 and Alr2 (aka DadX)), responsible for the anabolic and

73   catabolic functions respectively.

74   The catalytically active form of Alr is a dimer [9], due to the participation of

75   residues from both the monomers towards the formation of a functional active site. A narrow

76   passage from the exterior forms an entryway to the substrate binding cavity in the active site

77   and is lined by conserved residues, some of which have been demonstrated to orient the

78   substrate molecules during their entry into the active site [10, 11]. In Alr$_{Mtb}$, the substrate

79   binding cavity is a small, conical space gated by two tyrosine residues (inner gates), which

80   restrict the entry of substances into the active site [12]. Carboxylates such as acetate,

81   propionate and substrate analogs such as alanine phosphonate co-crystallize in the substrate

82   binding cavities of alanine racemases [13–15] and are suggested to regulate catalysis by

83   competitive inhibition, though the exact control mechanisms are not known [16].

84   Including the structure of Alr$_{Mtb}$ [12], there are around a dozen and a half

85   unique alanine racemase structures in protein databases [13, 17–23]. Though there has been

86   considerable interest in elucidating the detailed catalytic mechanism of D- to L-alanine

87   racemization in several organisms [5, 10, 24, 25], the regulatory aspects of catalysis suffer

88   from lack of research. In spite of the discovery of a plethora of inhibitors against pathogenic

89   Alr [26–28], only one of them has reached the market as a TB drug. This drug (D-

90   cycloserine) is a structural analog of D-alanine and binds to all PLP-containing enzymes non-

91   specifically, including those in the host, inducing toxic side-effects [29]. Current drug

92   discovery efforts focus on finding safer, selective, non-substrate inhibitors. Several inhibitors

93   of Alr are non-substrate leads, whose target sites on the enzyme are not known. Of these, five

94   were shown to be non-toxic to mammalian cells in a high-throughput screen for anti-

95 tubercular small molecule inhibitors [28]. Until now, there have been no studies concerning

96 the binding sites of these five drug-like leads (Fig 1**)** on the enzyme. Considering the

97 numerous hurdles in culturing *M. tb* and the urgency in developing novel drugs to contain the

98 superbug strains, we sought to determine the target sites of these leads through computational

99 studies.

Fig 1 Inhibitors shortlisted from Anthony et al., 2011 [28]. IUPAC names and molecular weights are shown for each lead inhibitor. All of the listed inhibitors obey Lipinski's 'rule of 5' characteristic of drug-like compounds and were shown to be non-cytotoxic to mammalian cells [28].

100 In recent years, normal mode analysis (NMA) has been widely used in

101 probing large-scale, collective motions of proteins and has been increasingly utilized to

102 characterize the dynamic aspects of enzymes [30–32]. Particularly, elastic network model

103 (ENM) based NMA has been useful in studying intrinsic dynamics of slow protein motions

104 over longer timescales [33, 34]. Computationally, the generation of elastic network models of

105 diverse protein conformations is less expensive compared to molecular dynamics (MD)

106 simulations [35]. In enzymes, ENM-NMA-predicted global motions represent biologically

107 relevant functional motions and have been shown to include local fluctuations such as loop

108 movements essential in catalysis [36]. We searched ENM-based Alr_{Mtb} conformations for

109 target sites of lead inhibitors through multiple, robust search algorithms by a blind docking

110 strategy (BD). BD remains a common choice in the discovery of novel, allosteric binding

111 sites [37, 38]. In conjunction with pocket search tools, BD is capable of identifying new

112 functional pockets on the target protein [39]. This strategy helped us in the successful

113 identification and validation of new pockets in Alr_{Mtb}. Further to the above investigations, a

114 comparative study of the intrinsic dynamics of Alr homologs with the help of a range of

115 computational tools helped us gain new insights into the regulatory aspect of D-alanine

116 synthesis.

Results / Discussion

All-atom normal mode analysis

The putative regulatory pockets are conserved across homologs The crystal structure of Alr$_{Mtb}$ is a kidney-shaped dimer, with two active site cavities opening on the convex side (Figs 2A, 2C and 2E) and two pockets located on the concave side (Figs 2A and 2D). Residues found to be missing (Fig 2B) in the crystal structure were from both internal and terminal regions. The internal stretches of missing residues (176−180 of subunit A and 266−280 of subunit B), pertained to the same region, i.e., the mouth region of the first active site cavity (Fig 2E).

Fig 2 Structure of alanine racemase from *Mycobacterium* tuberculosis. A. Molecular surface representation of the structure of alanine racemase (monomers A and B shown in green and cyan colours respectively). Magnified region shows the putative dimer interface groove (DIG) pocket on the dimer interface. B. Unresolved regions in the crystal structure of Alr indicated by different colours in the cartoon representation of the enzyme (missing N-terminus—yellow; missing C-terminus—blue; missing internal stretches—red). C. TIM-barrel of active site 2 showing the cofactor PLP (red sticks) covalently attached to the catalytic residue Lys44 (green sticks). Note that the active site is composed of residues from both monomers (B monomer shown in cyan colour and residues from A monomer are coloured green) D. Surface representation of the enzyme showing the putative regulatory sites (yellow) E. Surface representation of the enzyme showing tiny pockets (pink) flanked on either side by the active site cavities (red). (Due to the revision in UniProt sequence information, the residue numbers given in this work should be decremented by 2 in order to compare with the numbering provided in LeMagueres et al., 2005 [12]. For example, the residues, 176−180 in our work refer to residues, 174−178 in LeMagueres et al., 2005 [12]).

Alignment of the protein sequences of Alr homologs (Figs 3, S1 and S2) revealed highly similar residues in the newly identified regions (described later): dimer interface groove region (Fig 3B), putative regulatory sites (Fig 3C) and a short helix (Fig 3D). On the other hand, the N-termini of the homologous Alr were of different lengths and were dissimilar in sequence composition (Fig 3A). Despite the presence of terminus in their sequences, 8 of the crystal structures of the homologs were devoid of either the N-terminus (varied between 3−15 residues) or the C-terminus (varied between 1−6 residues) or both. Of

133 the remaining structures, eight were complete and showed disordered coils in their termini.

134 Both PSI-PRED (secondary structure predictor based on position-specific-scoring-matrices of

135 unique fold libraries) and Phyre2 (protein structure modeller based on a combination of *ab*

136 *initio* and template-based strategies) generated highly disordered coils in the terminal regions

137 (1–12 and 384–386) of *M. tb* racemase. Compared to the rest of the protein, the average B-

138 factor values in the termini of most of the alanine racemases displayed a marginal increase.

139 However, *Streptomyces lavendulae* (PDB ids: 1VFH, 1VFT, 1VFS), the closest structural

140 homolog of Alr$_{Mtb}$ (49% sequence identity), displayed 2–3 times higher values in the N-

141 terminal regions signifying a mobile terminus.

Fig 3 Structure-based multiple sequence alignment of alanine racemases. A. Aligned N-terminal region lacking definite secondary structures. B. Aligned DIG (dimer interface groove) pocket region. C. Aligned putative regulatory pocket and tiny pocket regions. D. Aligned region of the short helix (H2) residues implicated in the allosteric communication between the putative regulatory pockets and the active site residues. Shaded circles in different colours beneath the alignment show the residues in the corresponding pockets. (The alignment included sequences of *M. tuberculosis* (PDB code: 1XFC), *S. lavendulae* (PDB code: 1VFH), *S. coelicolor* (PDB code:5FAC), *C. glutamicum* (PDB code: 2DY3), *B. subtilis* (PDB code: 5IRP), *C. difficile* 630 (PDB code: 4LUT), *B. anthracis (Ames) (*PDB code: 2VD9), *S. aureus* (PDB code: 3OO2), *E. faecalis* (PDB code: 3E5P), *C. subterraneus subsp. tengcongensis* (PDB code: 4Y2W), *E. coli* (PDB code: 2RJG), *S. pneumoniae* (PDB code: 3S46), P. *fluorescens* (PDB code: 2ODO), *P. aeruginosa* (PDB code: 1RCQ), *A. baumannii* (PDB code: 4QHR), *B. henselae* (PDB code: 3KW3). X denotes cofactor 'PLP-Lysine' adduct, also called LLP).

142   Pockets were searched for in Alr$_{Mtb}$ as described in Methods after modeling

143 the missing residues into its crystal structure. Pocket analysis of the Alr$_{Mtb}$ dimer model

144 revealed the presence of two surface pockets beneath the terminal disordered coils. The

145 pockets were adjacent to each other at the dimer interface, one corresponding to each subunit

146 (Fig 2D). An examination of the crystal structures of the homologs revealed the existence of

147 such pockets, either exposed to the exterior (PDB ids: 2RJG, 3E5P, 1RCQ, 2ODO) or

148 sequestered beneath the terminal regions (PDB ids: 3S46, 2VD9, 4Y2W). Closed pockets

149 sequestered beneath the termini were spacious enough to accommodate typical drug-like

150   small molecules (combined Connolly's molecular surface volumes of the two pockets were

151   358 $\mathring{A}^3$, 900.4 $\mathring{A}^3$, 834 $\mathring{A}^3$ in 3S46, 2VD9, 4Y2W respectively). The inadvertent inclusion of

152   extra diagonal spaces in the pocket volume calculations of Alr_{Mtb} and the longer, lid-like N-

153   termini (Fig 3A) resulted in a single larger pocket in *M. tuberculosis* (combined Connolly's

154   molecular surface volume of the two pockets in the closed state of Alr_{Mtb} = 4355.9 $\mathring{A}^3$). In the

155   current discussion, the mere presence of these pockets across species is of significance.

156   Across homologs, two blocks of pocket residues (Fig 3C) were found to be conserved: region

157   292–295 in Alr_{Mtb} (part of a conserved motif GY[AG]DG) and region 370–375 in Alr_{Mtb} (375

158   is completely conserved, 372 is partially conserved). These regions were not exposed to the

159   solvent in the crystal structures with sequestered pockets.

160   Upon examination of the normal modes of Alr_{Mtb}, we observed that two low-

161   frequency normal modes, LF_8 and LF_{10} displayed the gradual closure of N-terminal regions

162   over the surface pockets. Of the 30 conformations of LF_8 (normal mode 8), 15 were non-

163   identical and described the enzyme transition between an open state (conformations 8 and 9)

164   and a closed state (conformations 23 and 24). The spectrum of conformations between the 9th

165   and 23rd showed the surface pockets at various stages of closure. Fluctuation plot of the

166   normal mode 8 (Figs 4A and 4B) revealed that the close-open movements of the terminal

167   regions are events, resulting from the closing and opening of the bulky N-terminal domains

168   of the enzyme. In other words, the terminal regions always moved along with the other

169   fluctuating regions in the mode 8 enzyme conformations. Such large-scale movements

170   involving entire domains were observed in every examined homolog (shown as cumulative

171   root mean square displacement (cRMS) plots in Figs 4C–4H). In the cRMS plots, fluctuation

172   patterns of Alr were extremely similar in the bacterial genera belonging to the same phylum

173   (Fig S3), viz., Actinobacteria (*M. tb* and *S. lavendulae*), Proteobacteria (*P.aeruginosa* and

174   *P.fluorescens*) and Firmicutes (*Streptococcus pneumoniae* and *Enterococcus faecalis*).

175   Overall, the fluctuation patterns, including the closure of the surface pockets (Movie S1)

176   occurring as part of N-terminal domain movements were similar in all of the examined Alr

177   homologs. In some of the homologs, the putative lid regions were shorter than those in *M. tb*.

178   In the closed conformations of such homologs, the pockets were not completely covered.

Fig 4 Fluctuations of bacterial alanine racemases. A. Putty cartoon view of fluctuations of Alr$_{Mtb}$ (Graph in panel B) mapped onto residues of the mycobacterial Alr structure colored from low to high values (0–13 Å as yellow to red). B–H. Plots of atomic displacements (derived from all-atom normal mode analyses calculated on NOMAD-Ref server) of bacterial alanine racemases, *M. tuberculosis* (PDB code: 1XFC), *S. lavendulae* (PDB code: 1VFH), *E. faecalis* (PDB code: 3E5P), *S. pneumoniae* (PDB code: 3S46), *E. coli* (PDB code: 2RJG), *P. aeruginosa* (PDB code: 1RCQ), *P. fluorescens* (PDB code: 2ODO), respectively along normal mode number 8 (LF$_8$). Collectivity measures and frequencies of normal mode are indicated for each organism.

179   Additionally, when the fluctuations of the first 10 normal modes were

180   clustered (ensemble NMA based on C-α coordinates), higher amplitudes were observed in the

181   termini of the homologs, *Streptomyces lavendulae* and *Caldanaerobacter subterraneus*

182   *subsp. tengcongensis* (Fig 5). The conformational location of the terminus could be

183   responsible for the observed oscillations, but that does not appear to be the case due to the

184   following points:

185   1.   Absence of definite secondary structures like α-helix or β-strand in the terminal

186   regions of the structures of homologs.

187   2.   Location of the disordered, flexible terminal regions over surface pockets in Alr

188   homologs.

189   3.   Presence of the pockets in one of the states in the crystal structures of homologs:

190   'sequestered' or 'exposed'.

191   4.   Closing and opening of the pockets during the intrinsic movements of the enzyme in

192   normal mode simulations.

193   5.   Docking of lead inhibitors to the surface pockets (discussed later).

194   Therefore, we conclude that the terminal fragments (especially the longer N-

195 terminal 1–12 residue region in *M. tb*) anchored over the surface pockets help in sequestering

196 small molecules, which are bound to the pockets. We propose that the two pockets in

197 question are putative regulatory sites and refer to them as RS pockets ('Regulatory Site'

198 pockets) throughout this study. As mentioned earlier, a few other regions in Alr_{Mtb} fluctuated

199 simultaneously with the close-open movements of the RS pockets:

200   1. Two new pockets (Fig 2A-magnified region) lying in the dimer interface junctions of

201   the enzyme were found to elongate and contract with concomitant increase and

202   decrease in their volumes. We name the pockets as DIG-A (Dimer Interface Groove

203   pocket of monomer A) and DIG-B (Dimer Interface Groove pocket of monomer B).

204   2. Two tiny pockets lying adjacent to each other, in-between the entrances of the active

205   site cavities (Fig 2E) exhibited close-open movements across the LF_8 ensemble of

206   conformations. The tiny pockets were high affinity binding sites of the native

207   substrate (alanine) in both the crystal structure and the ensemble conformations.

208   Charged residues, Arg373 and Glu367 of the tiny pockets were found to interact with

209   the substrate. Across the ensemble conformations, the tiny pockets remained closed

210   when the RS pockets were open. The tiny pockets and the RS pockets open on the

211   opposite faces of the enzyme and share a few residues (292, 373) between each other.

212   Other residues of the two pockets exhibit a unique arrangement in the sequence and

213   are placed side by side in an alternating fashion (Fig 3C). Consequently, the adjacent

214   residues in the structure belong to one of the two pockets and assume opposing states

215   at any given instant during the dynamics. Therefore, the tiny and the RS pockets may

216   be fulfilling opposing roles in regulation.

Fig 5 Cross species normal mode analysis of Alr. Ensemble normal mode analysis (eNMA) derived fluctuations (based on C-α coordinates) of the superimposed structures of the

homologs. Superimposition was based on a multiple sequence alignment of protein sequences of Alr homologs on Bio3D modules, which was then manually edited for errors (Text S1). The fluctuation profiles of the Alr homologs are stacked at off-set values of 0.5 between each other on the y-axis for clarity. Gaps in the graphs correspond to the gaps in the aligned sequences.

217 **N-terminal domain movements lead to closure of regulatory pockets** Normal modes of

218 frequencies less than 30 cm^{-1}, cover most of the amplitudes in atomic displacements [40, 41].

219 A previous study has shown that a single normal mode is capable of carrying a lot of

220 information on the conformational change of a given protein in terms of direction and pattern

221 of atomic displacements [42]. In Alr$_{Mtb}$, LF$_8$ adequately described movements between two

222 distinct states—one in which the putative RS pockets were open (Figs 6A, 6C) and the other

223 in which they were closed (Figs 6B, 6D). Nearly 67% of amino acid residues were found to

224 exhibit fluctuations (collectivity measure=0.6662). Among the all-atom normal mode

225 ensemble of 30 conformations defining LF$_8$, the closed and open conformations were both

226 found twice each (conformations 8 and 9 represented open states while conformations 23 and

227 24 showed closed states). The pockets started closing from conformation 20 onwards and

228 were fully closed in conformations 23 and 24. In the 23rd and 24th closed conformations,

229 active site cavities were twisted from their original positions (Movie S1). A closer

230 examination of Alr$_{Mtb}$ fluctuations showed that a twisted, hinge-like bending motion of the N-

231 terminal domains of each monomer, transformed the open state into the closed state (Fig 6).

232 The rigid body twist of the enzyme along the bulky N-terminal domains resulted in the

233 closure of the putative N-terminal lid region over the RS pockets. The closed and open state

234 fluctuations resembled the closing and opening of a door hinge with the two plates being the

235 N-terminal domains and the centre of the hinge being the far end of the C-terminal region.

236 Concordantly, higher deformation energies were seen in the pivot residues (96, 141,146, 261

237 and 263) of the dimer interface pocket region (DIG pocket region), part of which is the far C-

238 terminal region (Fig 7). Apart from this hinge-like region, the putative terminal lid regions,

239   the catalytic tyrosine and the short helix region showed higher deformation energy peaks

240   signifying greater local flexibility (Fig 7).

241   **Fig 6 Depiction of transition of LF$_8$ from an open to a closed conformation in Alr$_{Mtb}$.** (A)
242   and (B). Representation of the molecular surfaces of the open and closed states of the
243   mycobacterial alanine racemase. (C) and (D). Cartoon representations of the secondary
244   structures of open and closed states of Alr$_{Mtb}$. It can be noted that, the positions of helices (H3
245   and H4) have changed and the N-terminal putative lid-like regions are closed over the
246   regulatory pockets in the closed state.

Fig 7. Visualization of mycobacterial Alr structure on the basis of local flexibility. A plot of residue-wise cumulative deformation energies derived from the first 10 low frequency normal modes of mycobacterial alanine racemase (energies calculated on C-α NMA of Alr$_{Mtb}$ on a standalone implementation of Bio-3D package). The putty view of deformation energies mapped onto mycobacterial Alr structure is coloured from low to high values (2–421 kcal / mol as yellow to red).

247   Table 1 portrays the loss of the relative orientation of the active site residues

248   in space in the closed and open states as compared to that in the dimer model constructed

249   from the crystal structure of Alr$_{Mtb}$ (See Methods). In the closed state of the active site 1, one

250   of the inner gates, Tyr273, was 2.4 Å nearer to the other inner gate tyrosine, Tyr366,

251   compared to their positions in the open state. As stated before, these two tyrosines guard the

252   entrance of the substrate binding cavity. As discussed in LeMagueres et al. [12], these two

253   residues define an opening of 2.7 Å in the crystal structure and are suggested by LeMagueres

254   et al. [12] to move apart in order to permit entry and exit of small molecules in and out of the

255   active site cavity. The distance between the two inner gates in the dimer model of crystal

256   structure was 13.7 Å. This value is intermediate to the distance between the inner gates in the

257   closed state (13.0 Å) and the distance between the same residues in the open state (15.4 Å).

258   Thus, in the open state, the gates have moved further apart by 1.7 Å when compared to the

259   distance between them in the model of the crystal structure. At the same time, the inner gates

260   have come closer by 0.7 Å and are essentially closed in the closed state. Therefore, the active

261   site entrance is closed in the closed state. The above results provide a proof for the hypothesis

262   put forth by LeMagueres et al. that the inner gates must move apart prior to catalysis [12].

Table 1 Distances between C-α atoms of the substrate binding cavity residues[a]

Residue range	Active site 1				Active site 2			
	Distance between residues (Å)				Distance between residues (Å)			
	Open state (Conf. 8 and 9)	Closed state (Conf. 23 and 24)	Dimer model (Model of crystal structure with missing residues) (Conf. 16)	Difference between open and closed states (Å)	Open state (Conf. 8 and 9)	Closed state (Conf. 23 and 24)	Dimer model (Model of crystal structure with missing residues) (Conf. 16)	Difference between open and closed states (Å)
Tyr273 to Tyr 366 (Inner gates)	15.4	13.0	13.7	2.4	14.8	12.6	13.3	2.2
Tyr273 to Tyr48	19.3	18.4	18.1	0.9	18.7	18.1	17.6	0.6
Tyr273 to Met321	12.2	11.4	11.3	0.8	11.5	10.9	10.7	0.6
LYS44 to Tyr48	6.1	6.7	6.3	0.6	6.0	6.6	6.2	0.6
Tyr273 to Lys44	18.7	18.7	17.8	0	18.1	18.2	17.2	0.1
Cys320 to Tyr 366	10.0	9.5	9.5	0.5	10.1	9.6	9.6	0.5
Met321 to Tyr 366	8.1	7.7	7.7	0.4	8.0	7.7	7.7	0.3
Cys320 to Tyr48	12.6	12.9	12.4	0.3	12.7	13.1	12.5	0.4
Met321 to Lys44	9.5	9.2	9.0	0.3	9.5	9.3	9.0	0.2
Lys44 to Cys320	12.5	12.7	12.1	0.2	12.5	12.8	12.2	0.3
Tyr273 to Cys320	8.6	8.3	8.0	0.3	8.0	7.9	7.5	0.1
Met321 to Tyr48	9.1	9.3	8.9	0.2	9.2	9.4	8.9	0.2
Lys44 to Tyr 366	12.0	11.9	11.6	0.1	11.9	11.9	11.6	0
Tyr366 to Tyr48	7.4	7.5	7.2	0	7.4	7.5	7.2	0
Arg142 to Met321	14.2	13.7	13.5	0.5	13.8	13.3	13.1	0.5
Arg142 to Tyr366	21.0	19.4	19.6	1.6	20.7	19.2	19.4	1.5
Arg142 to Tyr273	10.4	10.0	9.6	0.4	10.4	10.2	9.7	0.2
Arg142 to Lys44	18.2	19.2	17.8	1.0	17.8	18.8	17.4	1.0
Arg142 to Tyr48	21.6	22.1	21.1	0.5	21.2	21.7	20.7	0.5

[a]calculated on PyMOL molecular graphics tool

263 **Ensemble NMA (eNMA) uncovers conservation of conformational dynamics across**

264 **homologs**

265 Comparative ensemble NMA (eNMA) results showed that flexibility

266 profiles were largely similar across anabolic alanine racemases (PDB ids: 1XFC/*M. tb*;

267 1VFH/ *Streptomyces lavendulae*; 2RJG/*Escherichia coli*; 3E5P/ *Enterococcus faecalis*;

268 4Y2W/*Caldanaerobacter subterraneus subsp. tengcongensis*; 3S46/*Streptococcus*

269 *pneumoniae*) as well as their catabolic counterparts (1RCQ/*Pseudomonas aeruginosa*;

270 2ODO/*Pseudomonas fluorescens*) (Fig 5). Regions differing in amplitudes between the two

271 types of racemases included alignment position 40 (higher amplitude in anabolic racemases)

272 and alignment position 65 (higher amplitude in catabolic racemases). For the complete

273 multiple sequence alignment and the alignment positions utilized in ensemble NMA of

274 homologs, refer to Text S1. Across the homologs, the conserved residues constituting the

275 invariant core of the enzyme were found clustered around the same amplitude (Fig S4-A).

276 Moreover, the alignment positions displaying partial conservation of residues also fluctuated

277 more or less to the same extent (Fig S4-B). Notably, conserved residue positions of the active

278 site residues (alignment positions 51, 281 and 329 corresponding to Alr$_{Mtb}$ residues 48, 273

279 and 321 resp.), putative RS pockets (alignment positions 77, 302 and 383 corresponding to

280 Alr$_{Mtb}$ residues, 74, 294 and 375 resp.), DIG region (alignment position 149 corresponding to

281 Alr$_{Mtb}$ residue, 144), 'Second cavity opposite to PLP' (refer to subsection 2 of section 3 for

282 details about this cavity) (alignment positions 140, 147, 179 and 330 corresponding to Alr$_{Mtb}$

283 residues 135, 142, 174 and 322) displayed nearly identical amplitudes across homologs.

284 In order to understand the nature of fluctuations between different regions of

285 the enzyme, we calculated dynamic cross-correlation maps (DCCM) of positional

286 fluctuations of amino acid residues (Fig 8). DCCM was based on the C-α coordinates of the

287 first 10 normal modes of Alr$_{Mtb}$ as against the all-atom NMA results (discussed in the

288   previous sections). Figs 8A–8C show that the correlations between residue pairs in Alr$_{Mtb}$ are

289   in excellent agreement with the all-atom NMA results (Figs 4A and 4B). Additionally,

290   DCCM provided insights into the directions of the movements between different regions. On

291   the whole, the region between residues 134 and 199, moved in opposite directions with

292   respect to the two flanking regions, 1–133 and 200–386. Both the completely correlated (Fig

293   8B) and completely anti-correlated (Fig 8C) regions revealed coupled networks of residues

294   that play a role in the close-open transition of the enzyme. For example, strong correlations

295   were seen in the following regions: putative lid region, catalytic lysine, RS pockets, tiny

296   pockets and the delimiting boundary residues of the substrate binding cavity. Similarly,

297   strong anti-correlations were seen between DIG residues (143, 144, 146 and 149), and the

298   regions listed in Table 2. Majority of the residues of the 'Second cavity opposite to PLP'

299   moved in opposite directions with respect to all the other regions of the enzyme. In agreement

300   with the all-atom normal mode results, the inner gates of the substrate binding cavity moved

301   with equal amplitudes in opposite directions. Thus, inner gates were moving away from each

302   other during the transition of the closed state to the open state. Noticeably, the movement of

303   the inner gates was occurring as a result of the movement and expansion of the entire active

304   site. Thus the DCCM of Alr$_{Mtb}$ derived from C-α fluctuations forms an additional proof for

305   the conclusion derived earlier from the all-atom NMA of Alr$_{Mtb}$ that the inner gates must

306   move prior to catalysis.

Fig 8. Correlation map revealing correlated and anti-correlated regions in Alr. A.
Heatmap showing the dynamic cross correlation map of C-α atom fluctuations derived from
an NMA of the first 10 low frequency modes of mycobacterial alanine racemase. Red regions
denote the completely correlated residue pairs (same period, same phase) while blue regions
denote the completely anti-correlated residue pairs (same period, opposite phase). White
regions correspond to residue pairs whose fluctuations are not correlated. The top and right
multi-colour bars on the heatmap correspond to various enzyme regions (coloured for easy
visualization). B. Strongly correlated motions between mycobacterial Alr residues. Red lines
portray the strong correlations between the regions mapped on the representative circle of a
mycobacterial Alr monomer. C. Strongly anti-correlated motions between mycobacterial Alr
residues. Blue lines portray the strong anti-correlations between the regions mapped on the

representative circle of a mycobacterial Alr monomer. D. Dynamic cross correlation map of residual fluctuations derived from an ensemble-NMA of the first 10 low frequency modes of alanine racemases from 8 different bacterial species. Only those correlations present in all the 8 structures are shown in the plot.

Table 2 List of newly discovered target sites and their putative interactions with the inhibitors

No.	Enzyme pocket	Ligand	Docking tools finding the enzyme-ligand complex	Enzyme residues involved in non-covalent interactions
1	Putative RS pocket	L2-05, L2-06	Vina, SwissDock	Ala45, Asp46, Glu74, Ala293, Gly374, Arg375, Arg378
		L2-10, L2-13	Vina, SwissDock, PatchDock	
2	DIG pocket region	L2-05, L2-06, L2-10	Vina, PatchDock, SwissDock	Asp137, Gly139, Asn141, Gly146, Gln149, Arg192, Gln199, Lys263, Arg266, Glu269, Gly270
		L2-13	Vina, SwissDock	
3	Tiny pocket	Alanine	Vina	Tyr292, Gly295, Ser299, Glu367,Ser371, Arg373
4	Second cavity opposite to PLP (cavity characterized in a previous study [12])	L2-05, L2-06, L2-10, L2-13	Vina, PatchDock	Trp90, Lys135, Arg142, His174, Asp322, Gln323

307   Both the correlation networks (Figs 8B and 8C) are each made of complete

308   graphs with well-connected nodes. The highly connected nodes in both the networks are hub-

309   like 'hotspots' which moved together either in the same or different directions. Therefore,

310   perturbing any of the sites corresponding to these hotspot nodes, by targeting them with small

311   molecules, would affect the regulatory control of catalysis, as all of these sites are involved in

312   the transition of the enzyme from an open to a closed state.

313   In order to understand the pattern of correlations across homologs, we

314   compared a residual fluctuation map showing only those correlations present in all the eight

315   homologs (Fig 8D) with the individual DCCM of each homolog (Figs 8A, S5–S11). We

316   found that the correlated and anti-correlated regions were nearly the same in all the maps,

317   pointing to an evolutionary conservation of dynamics across alanine racemases. This

318   conclusion is reiterated by the results of a comparative assessment of the normal modes of

319   pairs of homologs using similarity measures of dynamics including RMSIP (Root Mean

320   Square Inner Product) and Bhattacharyya coefficient (BC) (Table 3). RMSIP measures the

321   similarity of atomic fluctuations derived from normal modes between proteins, whereas BC

322   compares the covariance matrices obtained from the normal modes as elaborated in the

323   Methods section. For example, a thermo-stable Alr from *Caldanaerobacter subterraneus*

324   *subsp. tengcongensis*, which is a remote homolog of Alr_{Mtb} (sequence identity=28.6%) shows

325   99.84% similarity in dynamics, as measured by the Bhattacharyya coefficient. Though the

326   differences between the RMSIP scores were more pronounced than those of BC (Table 3),

327   the latter is generally considered to be a better index for assessing the similarity of dynamics,

328   as it incorporates eigenvalues. It is to be noted that RMSIP does not represent the energetic

329   separation between the modes in the sets [43]. Sequence and structural similarity measures

330   such as RMSD values scored lesser than dynamics similarity measures such as RMSIP and

331   BC values (Table 3), proving that the conservation of dynamics far exceeds the sequence and

332   structural conservation in alanine racemases.

Table 3 Measures of sequence, structural and dynamic similarity of alanine racemases across bacteria.

Alr homologs compared		Structural similarity (RMSD in Å)	% Sequence identity	% Sequence similarity	% Dynamic similarity[a][b]	
					RMSIP	BC
M. tb	*S. lavendulae*	1.86	46.7	61.8	84.09	93.8072
M. tb	*E. coli*	2.496	34.2	48.7	90.54	99.8376
M. tb	*E. faecalis*	2.168	34.0	47.1	92.11	99.8416
M. tb	*S. pneumoniae*	2.515	33.5	50.4	89.66	99.7842
M. tb	*C. subterraneus*	2.056	28.6	46.9	94.27	99.8444
M. tb	*P. aeruginosa*	2.531	34	47.6	90.02	99.8891
M. tb	*P.fluorescens*	2.471	34.4	48.7	90.83	99.9071
S. lavendulae	*E. coli*	2.223	36.1	49.2	85.08	94.5419
S. lavendulae	*E. faecalis*	2.298	34	50	83.66	92.3979
S. lavendulae	*S. pneumoniae*	2.363	37.2	52.7	84.65	93.6492

S. lavendulae	*C. subterraneus*	2.161	33.1	52.3	83.69	92.8465
S. lavendulae	*P. aeruginosa*	2.391	37.9	50	82.69	90.3598
S. lavendulae	*P. fluorescens*	2.338	37.1	47.2	84.61	92.6982
E. coli	*E. faecalis*	2.371	31.5	48.4	93.07	99.8565
E. coli	*S. pneumoniae*	2.512	30.9	50	93.85	99.929
E. coli	*C. subterraneus*	2.759	28.7	47.1	91.23	99.6967
E. coli	*P. aeruginosa*	1.658	46.8	62	94.74	99.9699
E. coli	*P. fluorescens*	1.65	46.3	62.6	96.38	99.9606
E. faecalis	*S. pneumoniae*	1.608	52.3	65.2	94.79	99.929
E. faecalis	*C. subterraneus*	2.123	33.9	56.7	94.51	99.8714
E. faecalis	*P. aeruginosa*	2.336	33.6	47.7	91.25	99.8367
E. faecalis	*P. fluorescens*	2.256	35.9	53	93.42	99.8653
S. pneumoniae	*C. subterraneus*	2.395	33.2	54.8	92.12	99.8599
S. pneumoniae	*P. aeruginosa*	2.472	35.7	51.5	93.35	99.8673
S. pneumoniae	*P. fluorescens*	2.394	36.7	49.9	94.52	99.8791
C. subterraneus	*P. aeruginosa*	2.723	33.2	51.3	89.86	99.676
C. subterraneus	*P. fluorescens*	2.607	31.9	51.6	91.56	99.7205
P. aeruginosa	*P. fluorescens*	0.6	73.4	83.8	98.41	99.9943

[a]Dynamic similarity values in terms of RMSIP and BC are given in percentages for comparison purposes (RMSIP or BC * 100)
[b]All similarity scores were calculated using only the C-α atoms of Alr homologs

333 **Exploring inhibitor binding sites on Alr$_{Mtb}$**

334 Large-scale docking simulations (For details, see Methods) between the lead

335 inhibitors (Fig 1) and the ensemble conformations (of the close-open transition) led to the

336 following conclusions:

337 **Substrate binding cavity is the primary target site of L2-04.** L2-04 penetrated the

338 substrate binding cavity in a majority of Vina and PatchDock complexes (122 Vina

339 complexes; 103 PatchDock complexes (Table S1)). The aromatic ring side of L2-04 was

340 often found in pi-stacking interactions between the inner gate residues, Tyr366 and Tyr273'

341 (residue labeled with a prime to indicate that it belongs to the opposite monomer) while its

342 tail formed hydrogen bonds with the cofactor in the substrate binding cavity of Alr$_{Mtb}$.

343 Substrate binding cavity measures 5.5 X 5.0 X 2.5 Å^3 and accommodates the substrate, L-

344 alanine. Many guest substrates, substrate analogues and inhibitors such as acetate,

345 propanoate, L-alanine phosphonate, lysine and D-cycloserine have been reported to occupy

346 this cavity in homologs [13–15, 17, 44]. In the crystal structure of a thermo-stable Alr of a

347   novel thermophile, *Caldanaerobacter subterraneus subsp. tengcongensis* [21], the substrate

348   is found between the catalytic residues, Lys40 and Tyr268 (equivalent to Lys44 and Tyr273

349   in Alr$_{Mtb}$) in the substrate binding cavity and forms hydrogen bonds (2.7 Å) with the catalytic

350   tyrosine. We found that the substrate, alanine (Fig S12) and L2-04 docked similarly, into the

351   active site of Alr$_{Mtb}$, through hydrogen bonds with the hydroxyl 'O' atoms of the catalytic

352   tyrosine, Tyr273' (bond length:2.79 Å) and the cofactor PLP (bond length:2.91 Å). In a few

353   complexes, L2-04 also formed salt bridges with the catalytic lysine (Lys44) of the enzyme. A

354   superposition of the docked Alr—L2-04 complex with the crystal structures of Alr-ligand

355   complexes from *Bacillus anthracis (Ames)*, *S. aureus*, *P. fluorescens* and *B.*

356   *stearothermophilus* (Fig 9A) uncovered conserved enzyme residues interacting with the

357   ligands across the complexes. Thus, the network of interactions of L2-04 with the active site

358   residues, especially with the conserved catalytic residues, Lys44, Tyr273 and the cofactor

359   PLP explains the effective abolition of racemization. Our results are in agreement with

360   previous reports [28] which suggest that L2-04 binds Alr$_{Mtb}$ reversibly. We propose that L2-

361   04 brings about reversible, competitive inhibition, where a competition set up between L2-04

362   and the substrate for the same space in the substrate binding cavity induces the establishment

363   of a dynamic equilibrium between the two species. The relative concentrations of the

364   substrate and L2-04, then decide the course of the catalytic process to proceed or terminate

365   intermittently.

Fig 9. Docking of inhibitors to different pockets in Alr$_{Mtb}$. A. Superposition of the
substrate binding cavities of Alr with various bound ligands. Substrate binding cavity
residues are shown as sticks and surfaces for reference. Ligands are represented as coloured
sticks: docked L2-04 (green sticks); crystallized alanine (pink sticks) in Alr of *C.
subterraneus subsp. tengcongensis*, PDB ID: 4Y2W; crystallized propanoate (orange sticks)
in Alr of *E. faecalis*, PDB ID: 3E5P; crystallized 4-amino-isoxazolidin-3-one (cyan sticks) in
Alr of *G. stearothermophilus*, PDB ID: 1XQL; crystallized acetate (yellow sticks) in Alr of
P. fluorescens, PDB ID: 2ODO. B. L2-05 docked to active site in the closed state of Alr$_{Mtb}$
(Vina pose 1, Binding affinity: −8.5 kcal/mol; PatchDock poses 1 to 10). Note the entry of
L2-05 (yellow sticks) into the 'Second cavity opposite to PLP' (cavity residues depicted as
red sticks and surfaces) and the formation of hydrogen bonds (blue lines) with the residues

Lys135, Arg142 in this cavity. Part of the inhibitor is still in substrate binding cavity (cavity residues depicted as green sticks and surfaces). C. L2-13 poses (yellow and orange spheres) docked to putative regulatory pockets in a cartoon representation of the closed form of Alr$_{Mtb}$. (Orange spheres represent Vina pose 7 / PatchDock pose 7 docked to RS pocket corresponding to monomer B—binding affinity −8.2 kcal/mol; Yellow spheres represent Vina Pose 8 / PatchDock poses 3, 8 docked to RS pocket corresponding to monomer A— binding affinity −8.1 kcal/mol).

366   **The target site extends into a cavity on the opposite side of PLP.** Unexpectedly, a sizeable

367   number of substrate poses bonded with residues of a second, larger (6.0 X 4.5 X 7.5 Å^3)

368   cavity adjacent to the active site on the opposite side of PLP. This cavity which has been

369   previously characterized in *M. tb* by LeMagueres et al. [12] is accessible from the active site

370   cavity. We refer to this cavity as 'Second cavity opposite to PLP' in this study. A very small

371   number of high affinity poses of L2-05, L2-06, L2-10 and L2-13 were found to interact with

372   the residues (Trp90, Lys135, Arg142 and His174) found in this cavity. Regardless of such

373   interactions, none of the inhibitors were capable of occupying this cavity completely due to

374   the presence of two or more ring systems in their bulky structures. Therefore, this cavity is

375   less suitable for accommodating ligands of higher molecular weights, such as L2-05, L2-06,

376   L2-10 and L2-13 (size range=370–470 daltons). Fig 9B shows L2-05 targeting a part of this

377   cavity. Because the poses of all the 4 inhibitors are often found stretched between the active

378   site and the second cavity, we believe that both the cavities are more suitable for ligands of

379   lower molecular weight such as alanine (89 daltons).

380   **Allosteric coupling to close-open movements are mediated by linker residues of a short**

381   **helix.** L2-05, L2-06, L2-10 and L2-13 (Fig 9C) targeted RS pockets multiple times through

382   consensus pocket residues that included arginine, alanine and glycine. A consistent feature in

383   all of the enzyme-inhibitor complexes was the interaction of the inhibitor with the pocket

384   residues, Asp46 and Arg378, both of which were charged and placed adjacent to each other.

385   Asp46 is situated on the same short helix (H2) of the active site TIM-barrel as the catalytic

386   lysine (K44). While Lys44 is present on the inside of the active site TIM-barrel, Asp46 is

387   present on the outside surface of the TIM barrel on the same short helix, but extending into

388   the RS pockets.

389   Superposition of the open and closed states, both of whose RS pockets were

390   bound with high affinity inhibitor poses (Fig 10A), clearly demonstrated the twisted active

391   site cavity in case of the closed state. Docked poses of the bound inhibitors were observed to

392   interact with charged RS pocket residues, Arg378 or Asp46 or both and such interactions

393   appears to be driving the pull experienced by the short helix, H2 (Y48-G47-D46-A45-K44),

394   seen in the normal mode motions. Such a movement of the short helix (Fig 10B) between the

395   active site cavity and the RS pocket leads to the expansion and contraction of the active site

396   cavities, as observed in the conformations of LF_8. The catalytic residue, Lys44, linked by a

397   covalent bond with the cofactor PLP on the inside of the TIM-barrels of the active site cavity

398   (Fig 2C), would be dragged along with Lys44 towards the periphery of the active site cavity.

399   As a result, the orientation of the catalytic residues would be lost. Tyr48, which walls the

400   substrate binding cavity on one side through its side chain, forms the other end of the short

401   helix and therefore would also be displaced, leading to the rearrangement of the substrate

402   binding cavity (Fig 10B). Thus, the dynamic interactions between the inhibitor and the

403   enzyme residues, viz., Arg378---Asp46 (R378---D46), is hypothesized to result in the short

404   helix motion and aid in the communication between the regulatory and the active sites. This

405   communication paradigm adequately explains the basis of signal (inhibitor binding to the

406   regulatory site) transduction from the environment, all the way to the interior of the enzyme

407   active site. The conservation of the charged arginine (Arg378) in the RS pockets across

408   homologs (20 of the 21 homologs) point to a pivotal role for this residue in inhibitor

409   interactions and regulation. In the short helix (YGDAK), the flanking residues, viz. the

410   catalytic Lys44 and the active site residue, Tyr48 are completely conserved while the three

411   middle residues showed equivalent substitutions (Glycine (G) by alanine (18/21), aspartic

412   acid (D) by asparagine (14/21 times), Alanine (A) by either glycine (3/21) or serine (2/21))

413   (Fig 3D). A novel measure for amino acid flexibility in peptides devised by Huang et al. [45]

414   ranks all the three middle residues of the short helix as highly flexible residues in the order,

415   Glycine > Serine > Alanine, Aspartic acid and Asparagine. This result is in agreement with

416   the need for higher conformational flexibility in the short helix residues in order to move

417   between the RS pocket and the active site upon inhibitor binding. Supporting the above

418   results, NMA studies show that deformation energies of the short helix residues are higher

419   than the surrounding structure, indicating higher local flexibility (Fig 7). Generally, in TIM-

420   barrel structures, there is a repetition of 8 alternating α helices and β strands. But, in case of

421   alanine racemases, the arrangement of the active site TIM-barrel is as follows: α1-β1-α2-α3-

422   β2-α4-β3-α5-β4-α6-β5-α7-β6-α8-β7-α9-α10-β8. It appears that the short helix H2 (α2), is an

423   additional insertion (most likely by the splitting of the original second helix into α2 and α3)

424   into the conventional TIM-barrel arrangement, the insertion event evolving probably later, in

425   order to carry out allosteric regulation.

Fig 10. Distortion of active site geometry in mycobacterial Alr. A. Residue positions in the superimposed open and closed states. L2-10 docked to RS pockets in the closed state shown as orange ball and sticks (Vina pose 6 / PatchDock pose 10; Vina binding affinity: −9.1 kcal/mol); L2-10 docked to RS pockets in the open state shown as green ball and sticks (Vina pose 6 / PatchDock pose 5; Vina binding affinity: −8.3 kcal/mol). B. Depiction of the short helix (H2) residue displacements upon the binding of a hypothetical ligand. Tyr48 (Y48 delimits the substrate binding cavity on one side) and Lys44-PLP adduct (K44-PLP adduct important in catalysis) being pulled away from their original positions towards the periphery of active site cavity upon the binding of a representative inhibitor to Asp46, a regulatory pocket residue.

426   The structures of L2-05, L2-06, L2-10 and L2-13 are novel and not analogs

427   of the substrate. Previous mass spectrometric analysis [28] have shown that three (L2-05, L2-

428   06 and L2-13) of these inhibitors bind to the enzyme irreversibly. In these experiments, the

429   binding mode of L2-10 could not be ascertained on account of an ambiguous peak profile.

430   The irreversible binding displayed by the three (L2-05, L2-06 and L2-13) inhibitors in the

431   mass spectrometry experiments when considered together with the docking results showing

432   RS pocket-bound high affinity inhibitor poses, suggests that allosteric interactions of the

433   inhibitors with the charged pocket residues (Asp46, Arg378) causes the irreversible inhibition

434   of the enzyme.

435   **Which of the enzyme states are catalytically active?** In the open state, the inner gates of

436   the active site entrance were the farthest from each other and were completely open. In

437   agreement, ensemble docking simulations showed that the substrate docked to the substrate

438   binding cavity in the open state (Active site 1) and intermediate conformations but not in the

439   closed state. In the open state, the substrate formed non-covalent interactions with the same

440   set of conserved active site residues as seen in the crystal structure of the thermo-stable Alr

441   (PDB ID: 4Y2W of *Caldanaerobacter subterraneus subsp. tengcongensis*) (Table 4). In

442   contrast, the active site entrance was twisted and closed in the closed state, rendering the

443   entryway (active site entrance) inaccessible. In such a shut active site cavity, catalysis is not

444   feasible. Therefore, we reason that the open state is catalytically active.

Table 4 Hydrogen bonds between L-alanine and alanine racemase residues

Structure	Type	Organism	Active site no.	Pose no.	Distance between L-alanine and PLP (Å)	Distance between L-alanine and Tyr273 or equivalent (Å)	Distance between L-alanine and Met321 or equivalent (Å)	Distance between L-alanine and Tyr292 or equivalent (Å)
Crystal structure	Reference structure	*Caldanaerobacter subterraneus subsp. tengcongensis*	1	-	-	2.67	2.56	2.8
			2	-	2.91	2.96	-	-
State 8 (open state)	Normal mode conformat	*Mycobacterium tuberculosis*	1	10	2.86, 3.06	-	3.22	-

	ions							
State 10			1	8	2.84, 3.09	-	3.19	-
State 13			1	8	2.87, 3.03	-	3.12	-
State 18			1	8	2.74	3.03	3.1	-
State 19			2	8	-	3.00	2.89	2.96, 3.04
State 20			2	9	-	2.99	2.94	2.93, 2.98
State 21			2	9	-	2.97	2.99	2.93, 2.95

Conclusion

445

446   Despite the increasing popularity of allosteric inhibitors as potential

447   therapeutic agents [46], the structural basis of the mechanism behind allosteric enzyme

448   inhibition remains virtually unknown. Alr_{Mtb} has more than one active site as is characteristic

449   of an allosteric enzyme. The presence of pockets at a location physically distant from the

450   active site together with the fact that the pocket volumes are optimum to house molecules in

451   the size range of natural regulators (activators or inhibitors), reinforces the theory of

452   allostery. The closure of the putative regulatory pockets, occurring as part of native dynamics

453   indicates that such an event is part of the catalytic process. Without the occupation of the

454   pockets by natural modulators during catalysis *in vivo*, it would be needless for the pockets to

455   be undergoing opening and closing movements. Moreover, docking of inhibitors to the

456   putative regulatory pockets, as evidenced by the ensemble docking results, fortify the

457   allostery proposal. Conservation of close-open enzyme movements across several Alr

458   homologs and the presence of 2 distinct states (closed and open RS pockets) in the crystal

459   structures of different organisms make a strong case for a generic allosteric mechanism of

460   regulation in alanine racemases.

461   In conclusion, computational investigations on alanine racemases aided in

462   identifying new, potentially druggable pockets on Alr_{Mtb}, bringing it back into focus as a

463   promising drug target. Many marketed TB drugs are orthosteric ligands and cross-react with

464   eukaryotic PLP-dependent enzymes limiting their usefulness as effective drugs. A

465   pharmacophore model based on the knowledge of scaffolds of the newly identified,

466   potentially druggable pockets will open doors to the discovery of novel, specific, allosteric

467   antimicrobials against *M. tb* with lesser side effects. The normal mode fluctuations between

468   the two different enzyme states is a testable computational hypothesis for experimental

469   studies aimed at deciphering the regulatory mechanism of alanine racemization. Correlation

470   of the short helix (Y48-G47-D46-A45-K44) motion to inhibitor binding at the allosteric site

471   detailed in this work offers a structural basis for allosteric site-to-active site communication

472   in Alr_{Mtb}. Inferences on the nature of inhibitor binding are in good agreement with

473   preliminary mass spectrometric experimental findings by Anthony *et al*. [28]. Mutation

474   studies of the conserved residues of the putative allosteric sites will help in determining the

475   role and essentiality of these new sites in the regulation of catalysis. The presence of

476   conserved residues in the putative regulatory pockets and the similarity in the fluctuation

477   profiles of Alr homologs points to a mode of regulation common to several bacterial species.

478   The disruption of this common regulatory mechanism by targeting the new pockets with a

479   single inhibitor in diverse bacterial pathogens is a prospective tip for the discovery of broad-

480   spectrum antibiotics in future drug discovery efforts.

481   **Methods**

482   **Preparation of *M. tb* alanine racemase structure**

483   The PDB [47] structure of mycobacterial alanine racemase (PDB id: 1XFC)

484   was retrieved and analyzed. Missing internal and terminal residues in each of the monomers

485 (10 N-terminal, 3 C-terminal residues) were constructed on Phyre-2 server [48] and a

486 standalone version of Modeller (v. 9.16) [49]. The revision of sequence information as per

487 the latest release of UniProt-KB [50] (UniProt id: P9WQA9), necessitated the splicing of 2

488 extra residues, 'methionine' and 'alanine' before the N-terminus prior to modelling. Initially,

489 Modeller optimized the model by the variable target function method (VTFM) with conjugate

490 gradients (CG). Subsequently, a preparatory molecular dynamics (MD) procedure with

491 simulated annealing (SA) (300 steps each of heating the model *in vacuo* from 150K to 1300K

492 and 1000 steps each of cooling the structure from 1300K to 300K) and a final, short

493 conjugate gradient optimization (43 steps) were carried out by Modeller. An elaborate energy

494 minimization of the model was additionally carried out by utilizing GROMACS [51] with

495 AMBER-99SB* [52] forcefield on the MDWeb server [53] (500 steps of energy

496 minimization of H atoms followed by 500 steps of energy minimization of the structure

497 restraining heavy atoms to their initial positions with a force constant of $500kj / mol*nm^2$).

498 The energy minimized model, thus obtained, was assessed on PDBSUM / PROCHECK [54,

499 55] and ERRAT [56] by analyzing Ramachandran plots [57] and other stereo-chemical

500 parameters. Secondary structures of the missing termini were predicted on the PSIPRED v

501 3.3 webserver [58].

Comparative analysis of alanine racemase homologs

503 A PSI-BLAST v.2.7.0 [59, 60] search for Alr homologs against the PDB

504 database yielded 16 distinct PDB structures in the last iteration (e-value threshold = 0.0001)

505 from 16 different organisms. Out of the 16, 14 were anabolic alanine racemases (Alr) and 2

506 were catabolic alanine racemases (Alr2/DadX). All the 16 sequences were aligned on the

507 ENDscript 2.0 web server [61] using the in-built Clustal Omega tool [62]. Upon examination

508 of the PDB structures of these 16 sequences, eight of them (*M*. *tuberculosis* (PDB code:

509   1XFC), *S. lavendulae* (PDB code: 1VFH), *E. faecalis* (PDB code: 3E5P), *E. coli* (PDB code:

510   2RJG), *P. aeruginosa* (PDB code: 1RCQ), *P. fluorescens* (PDB code: 2ODO), *S. pneumoniae*

511   (PDB code: 3S46), *C. subterraneus subsp. tengcongensis* (PDB ID: 4Y2W)) were found to be

512   completely resolved and were selected for all further analyses. Root Mean Square Deviation

513   (RMSD) between the aligned C-α coordinates of the atom pairs of the corresponding Alr

514   homologs were calculated in order to compare the similarity of native structures between any

515   two homologs.

All-atom normal mode analysis on individual Alr structures

517   Elastic network, Tirion-style normal mode models of alanine racemases

518   from *M. tb* (dimer model of PDB id: 1XFC) and 6 completely resolved Alr homologs viz.,

519   those of *Streptomyces lavendulae* (PDB id: 1VFH), *Escherichia coli* (PDB id: 2RJG, 2RJH),

520   *Streptococcus pneumoniae* (PDB id: 3S46), *Enterococcus faecalis* (PDB id: 3E5P, 3E6E),

521   *Pseudomonas aeruginosa* (PDB id: 1RCQ), *Pseudomonas fluorescens* (PDB id: 2ODO) were

522   generated on NOMAD-Ref web server [63] to explore collective, functionally relevant

523   movements in the enzymes. The first 10 non-trivial, low frequency normal modes were

524   generated for all atoms of the enzyme from each microorganism. As there is no provision

525   currently to include cofactors in NMA calculations, PLP was excluded in the NMA

526   calculations and was remodeled later into the NMA ensemble conformations prior to

527   docking.

528   NOMAD-Ref calculates the modes by considering a highly simplified,

529   quadratic potential energy between atoms that are assumed to be linked by a spring of

530   universal strength. The atom-pairs linked by such a spring of an arbitrary elastic constant of

531   100 kcal / mol / $Å^2$, were considered to be located <10 Å away in all the examined PDB

532   structures. Eigen frequencies of the normal modes were generated after weighting all the

533   interactions by $\exp\left(-(d_{ij}(0)/d_0)^2\right)$, where d_0 is the distance-weight parameter, which

534   effectively introduced a smoother "cut-off" value than the original Tirion model. Negative

535   eigenvalues were set to zero in the final output [63]. We obtained all our elastic models by

536   applying a distance weight parameter value of 6.8 Å for the elastic constant and utilized the

537   in-built Arnoldi iterative algorithm in order to diagonalize a sparse hessian matrix of n X n

538   dimensions (for example: n=17238 in *M. tb*). The amplitude of the protein movement was

539   controlled by fixing the average RMSD at 5 Å in output trajectories.

540   **Comparative normal mode analysis of Alr homologs**

541   In order to study the fluctuations in the conserved regions of the racemases,

542   ensemble-NMA elastic models of the superimposed invariant core of the enzyme across the 8

543   completely resolved Alr and DadX structures discussed before were generated on the eNMA

544   module of a standalone implementation of Bio3D v. 2.1-1 package [64]. In general, for all

545   downstream analyses, the data was filtered and only the first 10 low-frequency modes of each

546   homolog were included in the calculations. In ensemble-NMA calculations, multiple

547   sequence and structural alignment methods are utilized to analyse homologs. From these

548   alignments, equivalent atom positions across structure ensembles were selected and normal

549   mode vectors determined by calculating the effective force constant Hessian matrix K as,

550    $$\acute{K} = K_{AA} - K_{AQ}K_{QQ}^{-1}K_{QA} \qquad (1)$$

551   where K_{AA} represents the sub-matrix of K corresponding to the aligned C-α atoms, K_{QQ} for

552   the gapped regions, and K_{AQ} and K_{QA} are the sub-matrices relating the aligned and gapped

553   sites [65]. The normal modes of the individual structure in the ensemble can then be obtained

554   by solving the eigenvalue problem,

555    $$V^T \acute{K} V = \lambda \qquad (2)$$

556   where V is the matrix of eigenvectors and λ, the associated eigenvalues.

557   In order to analyze the flexibility profile of the mycobacterial racemase,

558   cross-correlations of residual fluctuations and deformation energy profiles were generated on

559   the filtered normal mode data. Across homologs, the alanine racemase motions along the

560   selected normal modes were compared with the help of similarity measures, viz., RMSIP and

561   Bhattacharyya coefficient.

562   **Flexibility measures to assess Alr$_{Mtb}$**

563   **Dynamic cross-correlation maps (DCCM)** The correlated motions undergone by C-α atoms

564   of Alr$_{Mtb}$ protein during the first ten low-frequency normal modes were calculated using the

565   Bio-3D DCCM module. The DCCM module generates a covariance matrix between residue

566   pair fluctuations, i, j covering the entire length of the mycobacterial enzyme [66]. A cross-

567   correlation coefficient is then calculated for each residue pair using the equation,

568
$$A_{ij} = \frac{\langle \Delta x_i \Delta y_j \rangle}{\sqrt{\langle \Delta x_i^2 \rangle}\sqrt{\langle \Delta y_j^2 \rangle}} \qquad (3)$$

569   Here, Δx_i and Δy_j correspond to the displacement of ith and jth residues from their mean

570   positions.

571   **Deformation energy profiles** Deformation energies were calculated from raw Eigen energies

572   and vectors of the first 10 normal modes of Alr$_{Mtb}$ (NMA based on C-α atoms) with the help

573   of Bio-3D modules. Deformation energy is a normalised measure of the energy contributed

574   by individual atoms of the model towards deformations of the structure [67].

575   **Similarity measures for comparison of enzyme motion across homologs**

576   **Root mean square inner product (RMSIP)** For comparing sets of normal modes, the root

577   mean squared inner product (RMSIP) of the first 10 low frequency modes are generally

578   included [68]. The RMSIP quantifies the similarity of the directions of these low energy

579   subspaces (the subset of low frequency normal modes) between any two given proteins.

580   RMSIP measures the cumulative overlap between all pairs of the 'l' largest eigenvectors, and

581   is defined as:

582
$$\text{RMSIP} = \left[\frac{1}{l}\sum_{i=1}^{l}\sum_{j=1}^{l}\left(v_i^A \cdot v_j^B\right)\right]^{1/2} \qquad (4)$$

583   where v_i^A and v_j^B represent the ith and j[th] eigenvectors obtained from protein A and B,

584   respectively, 'l' is the number of modes included (normally 10). The RMSIP measure varies

585   between 0 (orthogonal) and 1 (identical directionality).

586   **Bhattacharyya coefficient** The Bhattacharyya's coefficient (BC) provides a means to

587   compare two covariance matrices derived from NMA of two given proteins. For ensemble

588   normal modes, the covariance matrix (C) can be calculated as the pseudo inverse of the mode

589   eigenvectors:

590
$$C = \sum_{i=1}^{3N-6}\frac{1}{\lambda_i}v_i v_i^T \qquad (5)$$

591   where v_i represents the ith eigenvector, λ_i the corresponding eigenvalue, and N, the number

592   of C-α atoms in the protein structure (3N−6 non-trivial modes). As formulated by Fuglebakk

593   et al. [69], the Bhattacharyya coefficient can then be written as,

594
$$BC = \exp\left[-\frac{1}{2q}\ln\left(\frac{|\Lambda|}{(|Q^T C_A Q||Q^T C_B Q|)^{1/2}}\right)\right] \qquad (6)$$

595   where Q is a matrix in which columns are eigenvectors of the averaged covariance matrix

596    $(C_A + C_B)/2$, Λ is a diagonal matrix containing the corresponding eigenvalues, and q the

597   number of modes needed to capture 90% of the variance (cumulative eigenvalues) of Q. The

598   BC varies between 0 and 1, and equals to 1 if the covariance matrices, C_A and C_B are

599   identical.

600 **Docking simulations on the NMA ensemble conformations of Alr$_{Mtb}$**

601   Five potential drug-like inhibitors (Fig 1) from Anthony et al. (2011) [28]

602 were short-listed and subjected to docking simulations against the LF$_8$ ensemble of Alr$_{Mtb}$.

603 Prior to ensemble docking, the structures of the small molecules were prepared as elaborated

604 below: 3D structures of the inhibitors were analyzed for all possible tautomers, stereoisomers,

605 and major microspecies at pH 7 on the Chemicalize web server [70]. Lowest energy

606 conformers were generated on a standalone implementation of Chemaxon MarvinView

607 v.16.3.7.0 [71] by applying a Dreiding forcefield [72] at a diversity limit of 0.1 for a time

608 limit of 1000s. L-alanine, the native substrate of the enzyme was included in the ligand set.

609   In an attempt to achieve higher accuracy, we employed AD Vina v.1.1.2

610 [73], which has an advanced scoring function and conducts a more exhaustive sampling of

611 the possible binding modes. To minimize false positives, we applied two other docking

612 algorithms with diverse search methodologies, viz., SwissDock [74] and PatchDock [75] for

613 validation purposes. Although all the 3 docking algorithms employed robust methodologies

614 for searching as well as scoring, we based our analyses primarily on AD Vina results. This

615 strategy was adopted to nullify the bias exhibited by PatchDock and SwissDock towards

616 active site cavities (PatchDock favoured active sites over other locations, while SwissDock

617 clusters seldom entered active sites).

618   In addition to flexible inhibitors, we sought to introduce an implicit

619 flexibility in the enzyme, by utilizing an ensemble of conformations, which sampled relevant

620 functional movements. For this purpose, we selected 15 non-redundant conformations (9th to

621 23rd describing the 'close-open' transition) out of the 30 conformations defining the second

622 low frequency mode 8 (LF$_8$) of *M. tb* Alr, for all analysis. A total of 900 Alr-ligand

623 complexes describing 10 best binding poses for each enzyme-ligand pair resulted from AD

624   Vina runs. These results were validated by checking for identical target sites across 900

625   PatchDock complexes and approximately 3000 SwissDock clusters of binding poses with

626   significant energies.

627   In general, the input structures were prepared according to the specifications

628   of the docking software, retaining default values for all input parameters. In case of AD Vina,

629   exhaustiveness factor was increased proportionally in view of the larger volumes of the

630   search spaces probed, because of which, a minimum of around 100 runs were ensured for

631   each enzyme-ligand combination. A standalone version of AutoDock Tools 1.5.6 [76] was

632   used to prepare receptor and ligand structures in all AD Vina runs. AD Vina searches for

633   ligand poses with the help of an iterated local search global optimizer algorithm and scores

634   the results by an empirical X-score like function incorporating knowledge-based potentials.

635   PatchDock finds docking transformations with good shape complementarity and ranks them

636   based on geometric fit and atomic desolvation. SwissDock employs the EADock dihedral

637   space sampling algorithm and incorporates CHARMM22 [77] forcefield energies by way of a

638   multi objective scoring function using the FACTS solvation model [78]. Interactions of the

639   docked ligands resulting from all of the simulation runs were visualized on PLIP v.1.3.2 [79]

640   and an open-source version of PyMOL v.1.8 [80] (built and compiled from source code

641   downloaded from SourceForge [81]).

642   **Pocket analysis**

643   Normal mode conformations were investigated on the CASTp 3.0 [82] web

644   server for the presence of pockets. Consensus enzyme residues targeted by inhibitors across

645   pockets were gleaned by comparing the ligand-enzyme complexes with LigPlot+ v. 1.4.5

646   [83]. For all other input parameters, default values were retained. In general, custom perl

647   scripts were used for all file processing purposes.

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Author contributions

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886   **Supporting Information**

Figure S1. Multiple sequence alignment of Alr homologs from different bacterial species.
Figure S2. Sequence conservation profile of Alr$_{Mtb.}$
Figure S3. Dendrogram based on the identity of protein sequences of alanine racemases.
Figure S4. A. Scatter plot of the normal-mode derived fluctuations of conserved residues of

the superimposed homologs. This residue set corresponds to the invariant core of Alr$_{Mtb}$. B. Scatter plot of the normal-mode derived fluctuations of similar, structurally equivalent residues of the superimposed homologs. All calculations were performed using C-α coordinates of the superimposed invariant core of alanine racemase homologs on the ensemble NMA (eNMA) Bio-3D module.

Figure S5. Dynamic cross-correlation map of residual fluctuations of alanine racemase of *Streptomyces lavendulae*.

Figure S6. Dynamic cross-correlation map of residual fluctuations of alanine racemase of *Enterococcus faecalis*.

Figure S7. Dynamic cross-correlation map of residual fluctuations of alanine racemase of *Streptococcus pneumoniae*.

Figure S8. Dynamic cross-correlation map of residual fluctuations of alanine racemase of *Caldanaerobacter subterraneus subsp. tengcongensis*.

Figure S9. Dynamic cross-correlation map of residual fluctuations of alanine racemase of *Pseudomonas aeruginosa*.

Figure S10. Dynamic cross-correlation map of residual fluctuations of alanine racemase of *Pseudomonas fluorescens*.

Figure S11. Dynamic cross-correlation map of residual fluctuations of alanine racemase of *Escherichia coli*.

Figure S12. LigPlot comparison of the docked substrate pose (L-alanine) of Alr$_{Mtb}$ with homologous Alr complexes co-crystallized with alanine in *Caldanaerobacter subterraneus subsp. tengcongensis*.

Table S1. Results of docking simulation runs of substrate and inhibitors on the ensemble conformations of alanine racemase from *Mycobacterium tuberculosis*.

Table S2. Properties of pockets of NMA ensemble conformations as calculated on the CASTp server.

Text S1. Multiple sequence alignment of Alr homologs utilized in ensemble NMA (shown with alignment positions).

Movie S1. Secondary structure representation of normal mode number 8 in Alr$_{Mtb}$. N-terminal putative lid-like region is shown in red colour. Helices H3 (yellow) and H4 (violet) undergo displacements.
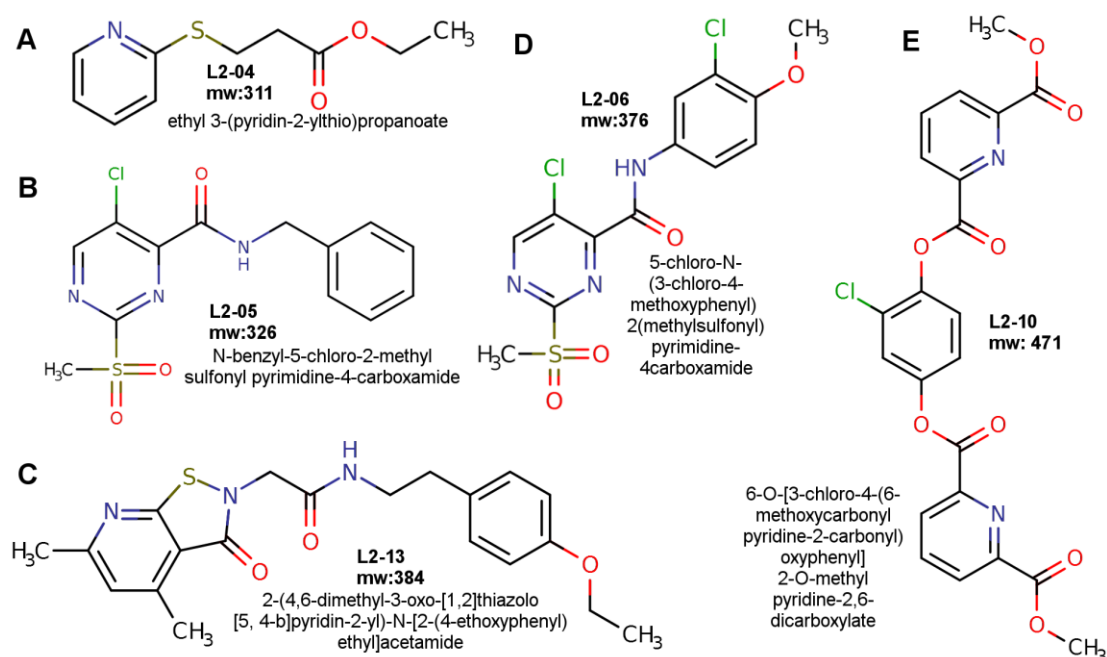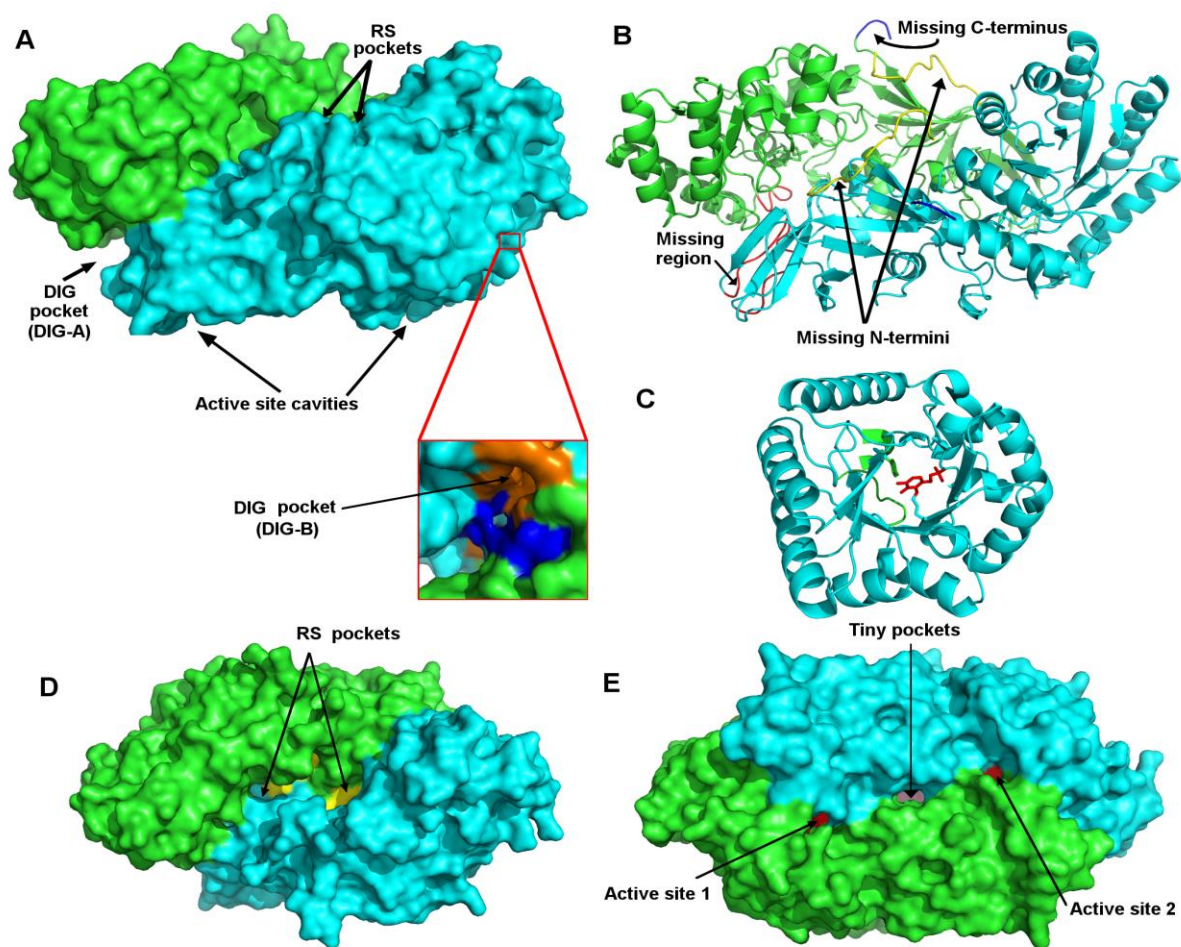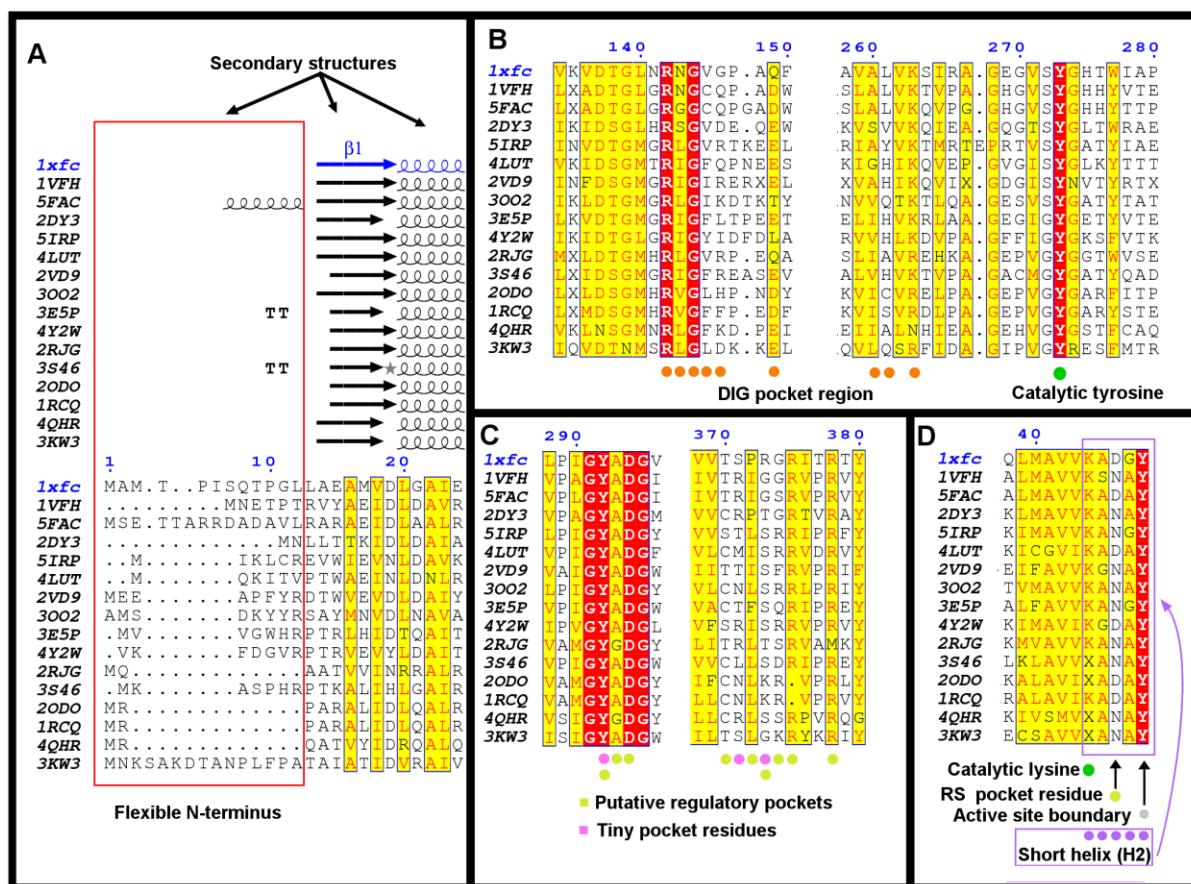
Figure 1

A

L2-04
mw:311
ethyl 3-(pyridin-2-ylthio)propanoate

B

L2-05
mw:326
N-benzyl-5-chloro-2-methyl
sulfonyl pyrimidine-4-carboxamide

C

L2-13
mw:384
2-(4,6-dimethyl-3-oxo-[1,2]thiazolo
[5, 4-b]pyridin-2-yl)-N-[2-(4-ethoxyphenyl)
ethyl]acetamide

D

L2-06
mw:376
5-chloro-N-
(3-chloro-4-
methoxyphenyl)
2(methylsulfonyl)
pyrimidine-
4carboxamide

E

L2-10
mw: 471
6-O-[3-chloro-4-(6-
methoxycarbonyl
pyridine-2-carbonyl)
oxyphenyl]
2-O-methyl
pyridine-2,6-
dicarboxylate

Figure 2

Figure 3

Figure 4

Figure 5

48
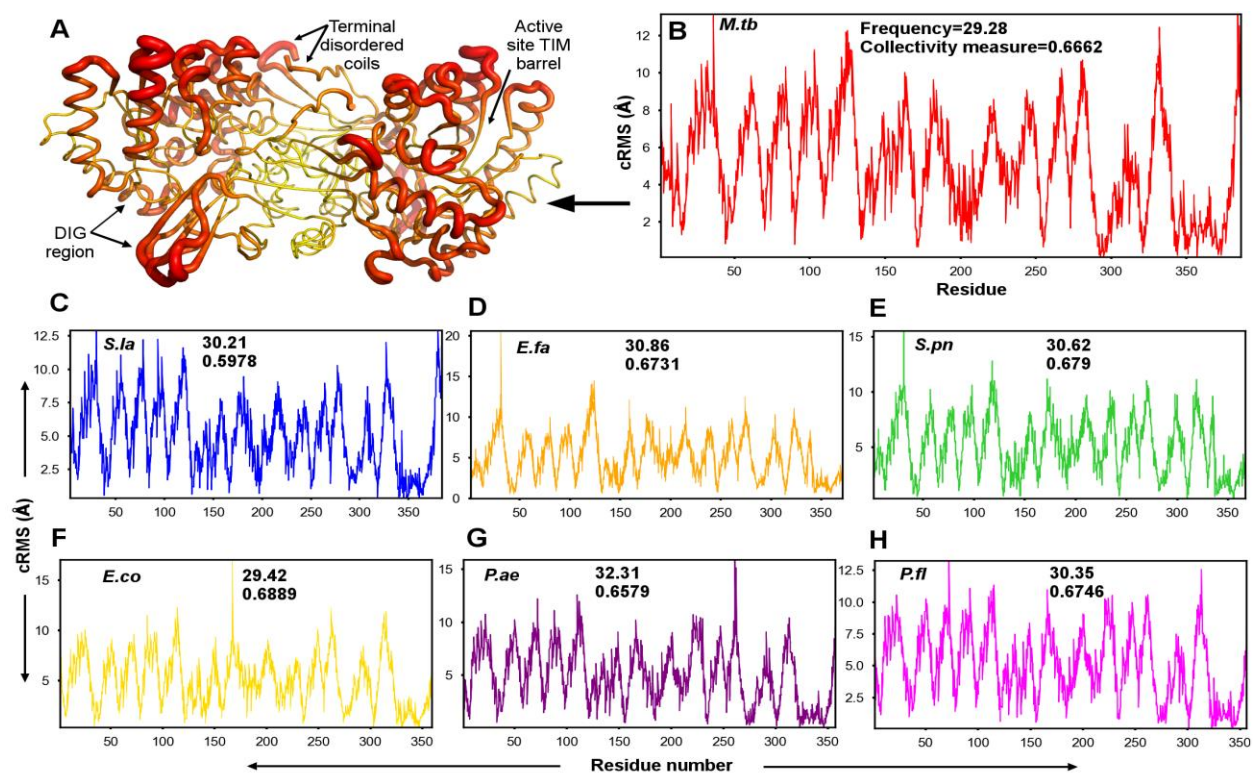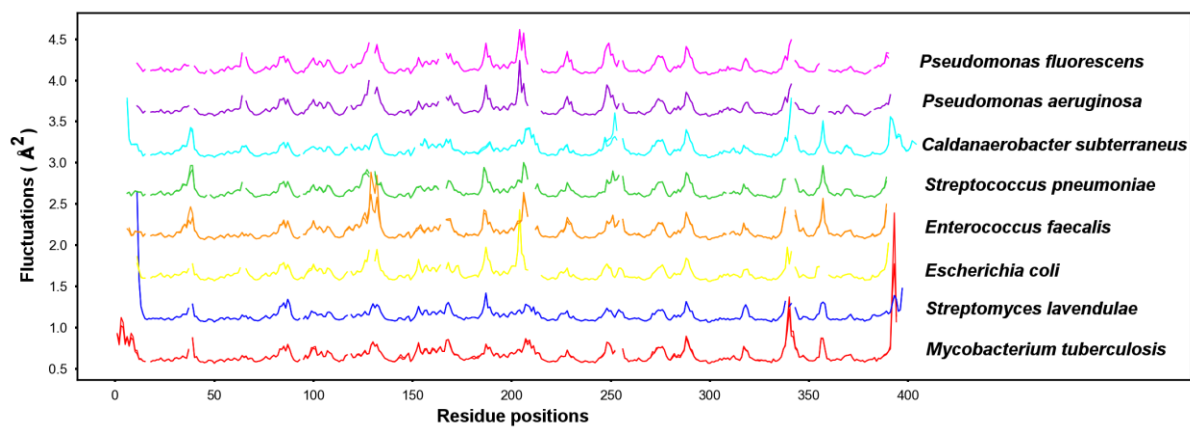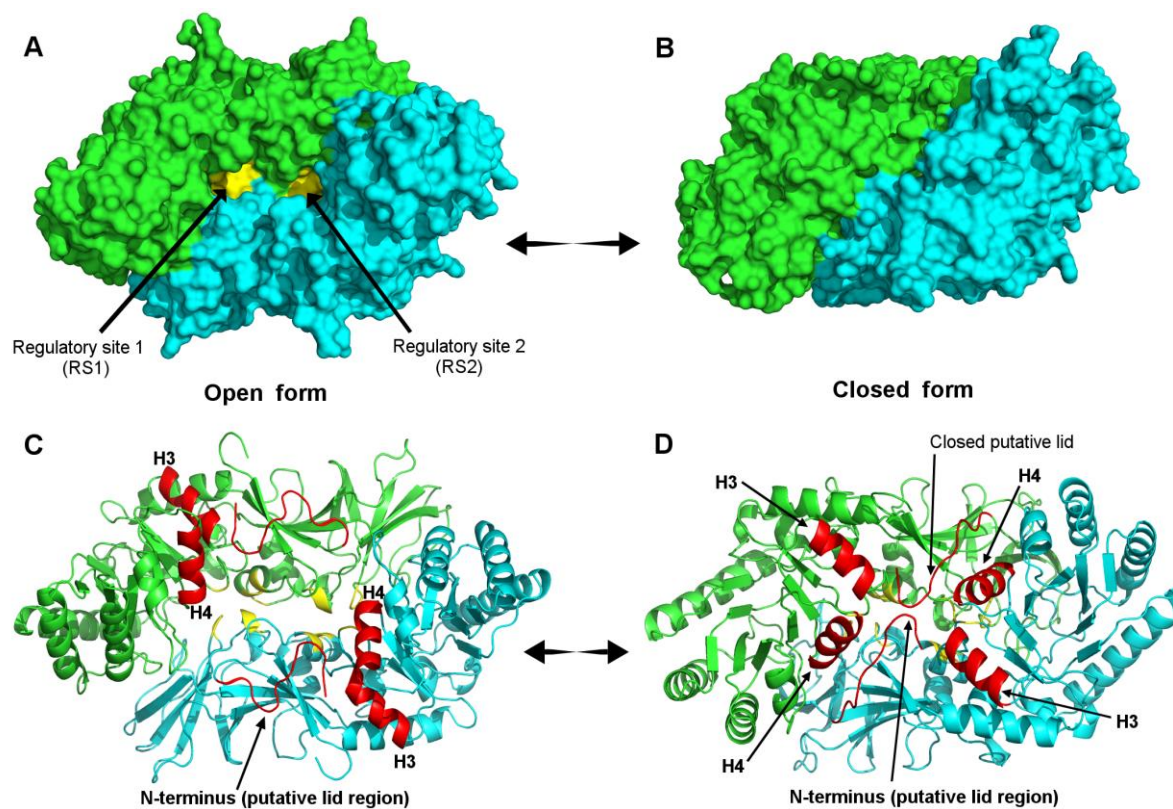
Figure 6

Figure 7

Figure 8

Figure 9

L2-13 docked to RS pockets in the closed conformation of *M.tb* Alr

Figure 10